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Award Number: DAMD17-99-1-9264

TITLE: Training Program in Breast Cancer Research at the University of Texas M.D. Anderson Cancer Center

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CONTRACTING ORGANIZATION: The University of Texas M.D. Anderson Cancer Center
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REPORT DATE: September 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | September 2004 | Annual Summary (1 Sep 1999 - 31 Aug 2004) |

**4. TITLE AND SUBTITLE**
Training Program in Breast Cancer Research at the University of Texas M.D. Anderson Cancer Center

**5. FUNDING NUMBERS**
DAMD17-99-1-9264

**6. AUTHOR(S)**
Mien-Chie Hung, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT (Maximum 200 Words)**

With the continuous funding from the US army/DOD (with an one-year no extension cost), the training program on breast cancer research at MDACC has had a successful fifth year. The training program has supported three pre-doctoral trainees last year and total 16 predoctoral and 16 postdoctoral fellows were supported by the program during the funding period. Each trainee has made notable progress as evidenced by publications and presentations at national meetings. Significant strides have been made within the scope of the original specific aims in the following research areas: therapeutic approaches for breast cancer through regulation of oncogene and tumor suppressor gene expression, and control of signal transduction and apoptosis; use of animals to understand the biology of breast cancer and to provide models for preclinical therapeutic and preventive studies; and The basis biology of breast cancer. The goal of the training program is to further the successful training of fellows who will develop research programs of their own which continue to tackle problems of breast cancer. Overall, it is a very successful program as evident from the numerous publications in peer-reviewed journals (listed in APPENDIX 4).

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| Breast cancer | 28 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

# FINAL REPORT OF DAMD 17-99-1-9264

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**FINAL REPORT of DAMD17-99-1-9264**

## INTRODUCTION

The Breast Cancer Training Program at the University of Texas M. D. Anderson Cancer Center is a multidisciplinary research program comprising students and faculty from twenty different departments. The ultimate goal of this training program is to provide support to aid the development of exceptional scientists in the field of breast cancer research. The predoctoral and postdoctoral trainees for the U. S. army/Department of Defense (DOD) training grant are chosen from the laboratories of all faculty involved in the entire Breast Cancer Research Program at MDACC which includes over seventy faculty members and twenty departments. The scope of the research conducted by the trainees includes a variety of topics related to breast cancer research. Some trainees are conducting research aimed at developing novel therapeutic approaches for breast cancer through regulation of oncogenes and tumor suppressor genes and control of signal transduction, apoptosis and DNA repair. Others are using animals to understand the biology of breast cancer and to develop models for pre-clinical therapeutic and preventive studies. Still others are studying molecular diagnostic/prognostic factors for breast cancer and developing novel preventive strategies for this disease. The trainees are involved in many departmental and interdepartmental events including journal clubs, group meetings, retreats, and seminars. Theses activities outside of the laboratory provide opportunities for the trainees to gain a truly multi-disciplinary perspective on their own research projects by communicating and collaborating with researchers from each department at MDACC involved in breast cancer research. The Breast Cancer Training Program at MDACC under the support of U.S. Army/DOD is well-established to producing superior investigators who will continue to explore the problems of breast cancer well into the future.

## BODY (STUDIES and RESULTS)

The support from the U.S. Army/DOD to develop the Breast Cancer Training Program at the University of Texas M. D. Anderson Cancer Center (UT-MDACC) has concluded with a great success in breast cancer research for the last five years. The Breast Cancer

Training Program at UT-MDACC was developed to provide support for exceptional young scientists training in laboratories that are part of the Breast Cancer Research Program (BCRP) at UT-MDACC. The program provides comprehensive interdisciplinary research training to each fellow. The program faculty is comprised of members who cover a full spectrum of breast cancer research, including investigators involved in basic, translation, clinical and population-based studies related to breast cancer. The strength of the training programs at UT-MDACC stems from strong interactions that take place between basic science researchers and clinicians in the exciting environment, which allows swift transfer of scientific discoveries from the laboratory to the clinic. Predoctoral and postdoctoral trainees at MDACC benefit from this unique environment and are able to gain unparalleled experience in multidisciplinary studies. The ultimate goal of this training program is to train predoctoral and postdoctoral fellows to become highly qualified breast cancer researchers who will develop programs of merit in breast cancer research.

The training program was instigated after approval of the U.S. Army/DOD training grant awarded to UT-MADCC. This program consists of over seventy faculty members from more than twenty departments at UT-MDACC involved breast cancer research. The program developed a nomination process that involved the participation of each faculty member in nominating individuals to serve on the U. S. Army Breast Cancer Training Grant Steering Committee. Following the nomination process each BCRP faculty member voted for his or her choices for members to serve the steering committee. This committee was formed to serve in the selection of U. S. Army Training Grant recipients, the mentoring of the selected fellows, and the monitoring of each fellow's research progress. This process resulted in the election of the following six faculty members to serve on the committee:

Mien-Chie Hung, Ph.D., Principal Investigator of U.S. Army/DOD Training Grant, Professor & Director, Breast Cancer Basic Research Program, and Chair, Department of Molecular and Cellular Oncology; Melissa Bondy, Ph.D., Associate Professor, Department of Epidemiology; Gabriel Hortobagyi, M. D., Professor & Chair, Department

of Breast Medical Oncology; Rakesh Kumar, Ph. D., Professor, Department of Molecular and Cellular Oncology; Janet Price, Ph. D., Associate Professor, Department of Cancer Biology; Dihua Yu, M.D., Ph.D., Associate Professor, Department of Surgical Oncology-Research

The steering committee commenced its activities by accepting proposals for the U. S. Army/DOD Breast Cancer Training Program Fellowships. When the U.S./Army/DOD training grant initiated five years ago, the steering committee has chosen four predoctoral and four postdoctoral trainees out of many candidates (ranging from a pool of 18-25 candidates) each year except the fifth year. Due to their exceptional qualifications, the U.S. Army Training Grant Steering Committee awarded an additional two postdoctoral trainees fellowships, and although designated as U. S. Army/DOD Training Grant Fellows, these individuals are supported by BCRP at UT-MDACC funding as reported previously. Thus, the awardees include four predoctoral trainees and two postdoctoral trainees who are supported by funds from the U. S. Army/DOD Training Grant each year (except the last year) and two more postodoctoral fellows who were supported by BCRP at UT-MDACC funding. There are total 16 predoctoral and postdocotal trainees received the award in the funding period (September 1, 1999 – August 31, 2003). Last yeast (September 1, 2003 – August 31, 2004) with an one-year no extension cost, there are three predoctoral trainees continued to be supported, Chi-Ping Day, Kan-Hsueh Lan and Dung-Fang Lee (please see below). The overall consensus is that the U. S. Army/DOD Training Grants in combination with the BCRP at UT-MDACC will not only provide each fellow with a excellent orientation to the various aspects of breast cancer research, but will also serve as a solid foundation in their careers.

**Annual summary from September 1, 2003 – August 31, 2004 (an one-year no extension cost)**

**Chi-Ping Day, M.S. - predoctoral trainee**

**The Role of Integrin-Linked Kinase (ILK) in HER2/*Neu*-Mediated NF-kB Activation Pathway**

HER2/*neu* activates the phosphatidylinositol-3 kinase (PI-3K)/Akt pathway, which activates IKKα/β, in turn activating NF-κB. Activation of nuclear transcriptional factor NF-κB by HER2/*neu* induces resistance to apoptotic stimuli, such as tumor necrosis factor (TNF) and chemotherapeutic agents. The integrin family of adhesion receptors also contributes in diverse ways to cell survival. Moreover, integrin has been shown to cooperate with HER2/*neu* to increased malignancy. Integrin linked kinase (ILK) is a transducer molecule directly downstream to integrin, thus dysregulated ILK expression or activity may contribute to oncogenic transformation.

To determine the effect HER2/*neu* has on ILK, we examined ILK kinase activity and protein expression in NIH3T3 and the HER2/*neu*-transformed NIH3T3 (3T3-HER2) cells. Our data suggest that HER2/*neu* induces ILK activity through PI-3K. To explore the role of ILK in HER2/*neu*-induced resistance to TNF-α treatment, we established two stable ILK-KD transfectant clones of 3T3-HER2 cells, namely 3T3-HER2-KD-1 and KD-2 (referred as KD-1 and KD-2). We found that ILK is required for HER2/*neu*-induced survival signaling, which leads to resistance to TNF-α and anoikis. To determine how ILK regulates NF-κB activity, we examine the effects of ILK-KD on the molecules upstream of NF-κB, and found ILK-KD inhibits NF-κB activity through the stabilization of IκB-α. Surprisingly, IKKα (α-IKKα) and IKKβ (α-IKKβ) protein levels were dramatically upregulated in 3T3-HER2 cells relative to parental NIH3T3 cells, and ILK-KD suppressed HER2/*neu*-induced IKKα and IKKβ protein levels in KD cells. These results indicate that HER2/*neu* induces the expression of IKKα and IKKβ through ILK. We also identified that HER2/*neu* upregulates the mRNA expression of IKKα and IKKβ through ILK. To explore whether Akt was involved in the ILK-induced expression of IKKα and IKKβ, we used a stable dominant-negative Akt (dnAkt) clone of 3T3-HER2 (3T3-HER2-dnAkt), and found that both ILK and Akt activation are required for HER2/neu-induced IKKα and IKKβ expression. To test whether the HER2/ILK/Akt pathway may activate transcription of IKKβ, we cloned a 567-bp 5'-upstream flanking region (5'UFR) of the mouse IKKβ gene. The putative IKKβ promoter was dramatically activated by HER2/*neu* and suppressed by dnAkt and ILK-KD, indicating that ILK and Akt were required for HER2/*neu*-mediated transcriptional activation of IKKβ.

**Kan-Hsueh Lan, M.D. – predoctoral trainee**

**Peptide/Protein Delivery System Targeting erbB2-overexpressing Breast Cancers**

Overexpression of erbB2 was found in ~ 30% of breast cancers and shown to correlate with the number of lymph node metastases and poor prognosis of the patients. *erb*B2 gene overexpression leads to enhanced metastatic potential and increased chemoresistance of breast cancer cells, hence poor clinical outcome of patients. Therefore *erb*B2 should serve as an excellent target for the development of novel cancer therapies. The major goal of this study is to explore the possibility of developing a TAT-based targeted delivery system to introduce specific therapeutic peptides into erbB2-overexpressing breast cancer cells. ErbB2 overexpression can activate Stat3 in multiple breast cancer cell lines, which contributes to ErbB2-induced cell transformation and progression. Therefore, Stat3 can serve as an excellent alternative down-stream target of ErbB2. One Stat3 inhibitory peptide PY(p)L has demonstrated efficient inhibition on Stat3 DNA binding and growth of Stat3-activated cancer cell. We decided to attach the PY(p)L Stat3 inhibitory peptide (αStat3)to the erbB2-targeting TAT-AHNP vehicle as the alternative approach.

Since we and others found that ErbB2 overexpression can activate Stat3 in multiple breast cancer cell lines, which contributes to ErbB2-induced cell transformation and progression, Stat3 can serve as an excellent alternative down-stream target of ErbB2. Another Stat3 inhibitory peptide PY(p)L has demonstrated efficient inhibition on Stat3 DNA binding and growth of Stat3-activated cancer cell, we decided to attach the PY(p)L Stat3 inhibitory peptide (αStat3)to the erbB2-targeting TAT-AHNP vehicle as the alternative approach. The synthesis of fluoresceine-TAT-AHNP-PY(p)L (TAT-AHNP-αStat3) was successful. We tested the translocation ability of TAT-AHNP-αStat3 peptide. As shown in Fig. 1 in APPENDIX 2, TAT-AHNP-αStat3 successfully translocated into 435.eB cells (green fluorescence) in vitro. Furthermore, the signals are stronger in 435.eB compared to those in the MDA-MB-435 cells, indicating that TAT-AHNP-αStat3 demonstrates the similar erbB2-targeting ability as TAT-AHNP does. Next we evaluated the growth inhibition effect of TAT-AHNP-αStat3 *in vitro*. Different concentrations (5, 50, or 100 mM) of peptides were applied to MDA-MB-435 or 435.eB cells. The viability of cells was measured after 54 and 102 hours (Fig. 2 of APPENDIX 2) of peptide treatment,

respectively, using MTS assay. With 50uM of TAT-AHNP-αStat3 treatment, MDA-MB-435 showed inhibited cell growth (~50%) while 435.eB showed a more dramatic inhibition (~65%; $p<0.05$). On the other hand, the control peptides (TAT-AHNP and AHNP) didn't significantly inhibit cell growth of either cell line ($p<0.005$). Therefore, we conclude that TAT-AHNP-αStat3 has a cell growth inhibition effect, which is conferred by the αStat3 domain (TAT-AHNP-αStat3 versus TAT-AHNP). Also, the greater extent of growth inhibition in 435.eB compared to that in MDA-MB-435 indicates that TAT-AHNP-αStat3 peptide preferentially targets the erbB2-overexpressing cells.

To evaluate if TAT-AHNP-αStat3 can also inhibit the growth of breast tumors *in vivo*, we established MDA-MB-435 and 435.eB tumor xenografts in the mammary fat pads (MFPs) on either side of 24 female SCID mice. The mice were randomly divided into two groups (12 mice in each group), and when the tumors became palpable (~30mm$^3$ of volume), we began to intra-peritoneally (i.p.) inject each group of mice with 15 nmole/mouse of TAT-AHNP-αStat3 or TAT-AHNP (control) peptides, respectively, three times per week. Tumor diameters were serially measured with calipers and tumor volumes were calculated using the follow formula: volume = width$^2$ × length/2. In the MDA-MB-435 xenografts (Figure 3 left), TAT-AHNP-αStat3 did not show an increased growth inhibition compared to TAT-AHNP. However, TAT-AHNP-αStat3 treatment significantly inhibited the growth of 435.eB xenografts (Fig. 3 right of APPENDIX 2) in comparison with TAT-AHNP treatment. There are some possible explanations for this differential inhibition of tumor growth. First, due to the erbB2-targeting ability of TAT-AHNP domain, the majority of TAT-AHNP-αStat3 peptides might accumulate in the 435.eB xenografts, while only a small fraction of peptides homed to MDA-MB-435 xenografts, resulting in this differential inhibition. Second, the activation level of Stat3 in 435.eB is much higher than that in MDA-MB-435, therefore, 435.eB cells could be more susceptible to the inhibitory effects of TAT-AHNP-αStat3 peptides.

In summary, these data demonstrated that we have successfully developed a TAT based peptide delivery system targeting specifically ErbB2-overexpressing breast cancer cells both *in vitro* and *in vivo*. Using this delivery system, we successfully delivered Stat3-

blocking peptide to ErbB2-overexpressing breast cancer cells both *in vitro* and *in vivo*. This TAT-AHNP-αStat3 peptide achieved growth inhibition of breast cancer cells both *in vitro* and *in vivo*. Therefore, this ErbB2-targeting peptide delivery system holds tremendous promise in the treatment of ErbB2-overexpressing cancers with its potential to deliver various other bio-chemically amendable therapeutics.

**Dung-Fang Lee, M.S. – predoctoral trainee**

**A potent proapoptotic hybrid gene, tBid and Bax/Bak, in breast cancer**

Bcl-2 family proteins are important modulators of pro-apoptosis (e.g. Bid, Bak and Bax) and anti-apoptosis (e.g. Bcl-2 and Bcl-$X_L$). In order to induce apoptosis, the "pro-apoptotic ligand"-tBid, i.e. the caspase-activated (truncated) form of Bid, induces its "pro-apoptotic receptor"-Bak translocating from the cytosol to the outer membrane of mitochondria, resulting in oligomerization of Bak/Bax and releasing cytochrome c from the mitochondria. Bcl-2/Bcl-$X_L$ inhibits the tBid-induced apoptosis by suppression of tBid and Bax translocation, and Bak/Bax oliogomerization on the outer membrane of mitochondria. Overexpression of Bcl-2/Bcl-$X_L$, which usually occurs in primary breast cancer, may contribute to breast tumor progression and increase the resistance to the apoptotic activity induced by tBid. To circumvent the higher affinity of Bcl-2/Bcl-$X_L$ toward the wild type tBid, we generate the hybrid gene, *tBid-Bax* that might possess a more potent pro-apoptotic activity than that of wild type tBid alone in breast cancer therapy regardless of Bcl-2/Bcl-$X_L$ status.

We have successfully constructed hybrid genes (tBid-Bax; tBid-Bak) (Fig. 1a of APPENDIX 3). In addition, a linker $(GGGS)_4$ was used to provide more flexibility of these two genes (*tBid* and *Bax*; *tBid* and *Bak*) and allow these two molecules could interact with each other. Furthermore, we construct several hybrid genes including pcDNA3 tBid-Bax and pcDNA3 tBid-Bak (Fig. 1b of APPENDIX 3) driven by CMV promoter. To investigate these hybrid genes contain the ability of prevent the abrogation by Bcl2 or Bcl-XL, we use GST-Bcl2 to pull down radiolabeled hybrid genes. Firstly, we expressed the GST-Bcl2 and GST-Bcl-$X_L$ in BL21 cells (Fig. 2a of APPENDIX 3) and purify them by Glutathione-Sepharose 4G beads (Amersham Pharmacia Biotech).

Secondly, hybrid genes (*tBid-Bax; tBid-Bak), tBid, Bax*, and *Bak* are expressed and radiolabeled by *in vitro* transcription and translation Kit (Promega) (Fig. 2b of APPENDIX 3). Thirdly, GST-Bcl2 is incubated with these radiolabeled products and pulled down by Glutathione-Sepharose 4G beads, separated by SDS-PAGE, and the results are detected by X-ray films. As shown in Fig. 2c of APPENDIX 3, the ability of pulling down tBid-Bax by GST-Bcl2 is dramatically decreased compared with the ability of pulling wild type Bax by GST-Bcl2, suggesting the tBid-Bax prevents the association with Bcl2 but Bax does not. In addition, another type of hybrid gene Bad2SA-Bax, (replacing tBid with Bad2SA, in which two AKT phosphorylation sites were mutated to Ala) is also examined whether it could bind with Bcl2 or not. As shown in Fig. 2d of APPENDIX 3, Bad2SA-Bax still interacts with Bcl2 and is pulled down by Sepharose beads. Our results demonstrate that *tBid-Bax* has a lower binding affinity with Bcl-2/Bcl-$X_L$ than wild type tBid/ Bax has in GST-pull down assays.

To clarify whether the hybrid gene (*tBid-Bax*) has better therapeutic effects compared with wild type proapoptotic genes such as *tBid* and *Bax*, we cotransfected these genes with a luciferase reporter into several different cell lines and examined their killing effects. As shown in Fig. 3 of APPENDIX 3, tBid-Bax has a better killing effect than wild type tBid/ Bax does in breast cancer MCF7 cell line. Moreover, the killing effect of tBid-Bax is comparable with that of Bax in MDA-MB-435, HEK-293, and B16 cells. These results demonstrate tBid-Bax has a potent proapoptotic activity. Additionally, we also study whether tBid-Bax could prevent the abrogation by Bcl2, we transfected these genes into MDA-MB-231 Bcl2-overexpressing stable cell lines (No. 4 and No. 5) (Fig. 4a of APPENDIX 3) and parental cells and to study the proapoptotic effect in these cell lines. As shown in Figs, 4 b, c, and d of APPENDIX 3, the killing effects of tBid-Bax and Bax are comparable. tBid-Bax exhibits a better killing effect than wild type Bax does in Bcl2 stable transfectant of MDA-MB-231 cells (No. 4 and No. 5), revealing that tBid-Bax has a potential to escape the inhibition of Bcl2 but wild type tBid and Bax have not these potentials. Thus, from the cytotoxic study in the Bcl-2 stable transfectant breast cancer cell lines reveals that tBid-Bax not only overcomes anti-apoptotic activity of Bcl-2 but also exhibits a stronger growth-inhibitory activity than that of wild type tBid. Taken

together, our newly developed hybrid gene, *tBid-Bax* could be a potent therapeutic gene for breast cancer containing overexpression of Bcl-2/Bcl-$X_L$.

**Key Research Accomplishment**

- Our study identifies a novel signal cascade, HER2/neu → PI-3K → ILK → Akt → IKKα/IKKβ transcription, which links the HER2/*neu*-mediated resistance to anoikis- and TNF-α-induced apoptosis.
- We have successfully developed a TAT based peptide delivery system targeting specifically ErbB2-overexpressing breast cancer cells both *in vitro* and *in vivo*.
- We have successfully delivered Stat3-blocking peptide to ErbB2-overexpressing breast cancer cells both *in vitro* and *in vivo*. This TAT-AHNP-αStat3 peptide achieved growth inhibition of breast cancer cells both *in vitro* and *in vivo*.
- *tBid-Bax* has a lower binding affinity with Bcl-2/Bcl-$X_L$ than wild type tBid/ Bax has in GST-pull down assays.
- Our results suggest that the hybrid tBid-Bax is more potent than tBid and Bax are as a therapeutic gene for breast cancer with overexpression of Bcl-2/Bcl-$X_L$.

**Reportable Outcomes**

**September 1, 2003 - August 31, 2004**

- **Makino K, Day CP**, Wang SC, Li YM, Hung MC. Upregulation of IKKα/IKKβ by integrin-linked kinase is required for HER2/neu-induced NF-κB antiapoptotic pathway. *Oncogene* 6:3883-7,2004. (APPENDIX 1)
- **Summary of trainees' publication: September 1, 1999 - August 31, 2004**
  **(in alphabetical order, please see APPENDIX 4 for detail)**

| Trainee Name | # of publication |
|---|---|
| Day, CP | 2 |
| Klos, K | 2 |
| Lan, KH | 1 |
| Lee, DF | 1 |
| Li, Y | 1 |

| | |
|---|---|
| **Liao, Y** | **2** |
| **Lim, SJ** | **2** |
| **Makino, K** | **6** |
| **Najafi, SMA** | **1** |
| **Wang, RA** | **4** |
| **Wang, X** | **1** |
| **Wen, Y** | **6** |
| **Yu, Z** | **1** |
| **Zhou, BH** | **3** |
| **Zhou, C** | **2** |
| **Zu, JJ** | **1** |

## CONCLUSION

In conclusion, the U.S. Army/DOD Breast Cancer Research Training Program at the University of Texas M. D. Anderson Cancer Center has had a great success. Each U.S. Army/DOD Training Grant recipient has benefited from the multidisciplinary program as evidenced by significant progress in their respective research projects and an outstanding publication record. The APPENDIX 4 is the list of publication in the entire funding period, which have been made reported previously (except the one reported in this year) and within the scope of the original specific aims in the following research areas: 1) Therapeutic approaches for breast cancer through regulation of oncogene and tumor suppressor gene expression, and control of signal transduction, apoptosis, and DNA repair; 2) Use of animals to understand the biology of breast cancer and to provide models for preclinical therapeutic and preventive studies; 3) Novel preventive strategies for breast cancer; 4) Population-based studies on breast cancer; 5) Molecular diagnostic/prognostic factors for breast cancer; and 6) The basis biology of breast cancer. We greatly appreciate this unique opportunity that is fully supported from the U.S. Army/DOD Breast Cancer Research Program to allow UT-MDACC prospering in training scientists in the field of breast cancer research.

## APPENDICES

## APPENDIX LIST

1. Makino K, Day CP, Wang SC, Li YM, Hung MC. Upregulation of IKKα/IKKβ by integrin-linked kinase is required for HER2/neu-induced NF-κB antiapoptotic pathway. *Oncogene* 6:3883-7,2004.

2. Three Figures and Figure Legends
   Kan-Hsueh Lan, M.D. – predoctoral trainee
   Peptide/Protein Delivery System Targeting erbB2-overexpressing Breast Cancers

3. Four Figures and Figure Legends
   Dung-Fang Lee, M.S. – predoctoral trainee
   A potent proapoptotic hybrid gene, tBid and Bax/Bak, in breast cancer

4. List of Publication (September 1, 1999 – August 31, 2004).

Oncogene (2004), 1–5

www.nature.com/onc

SHORT REPORT

# Upregulation of IKKα/IKKβ by integrin-linked kinase is required for HER2/*neu*-induced NF-κB antiapoptotic pathway

Keishi Makino[1,2,4], Chi-Ping Day[1,3,4,5], Shao-Chun Wang[1], Yan M. Li[1,3,6] and Mien-Chie Hung*[,1,2]

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**Constitutively active HER2/*neu* activates nuclear factor kappa-B (NF-κB) in cells and induces their resistance to apoptotic stimuli such as tumor necrosis factor-α (TNF-α). Here, we show that integrin-linked kinase (ILK), the crucial signal transducer in the integrin pathway, is involved in HER2/*neu*-mediated activation of NF-κB. Expression of HER2/*neu* increases ILK activity. Blocking ILK activity with a kinase-deficient mutant ILK (ILK-KD) inhibits NF-κB activation and sensitizes HER2/*neu*-transformed cells to TNF-α-induced apoptosis. Stable expression of ILK-KD in HER2/*neu*-transformed cells suppressed Akt phosphorylation and the expression of IκB kinase α and β (IKKα and β) at both the protein and mRNA levels, preventing IκB-α degradation and NF-κB activation. Furthermore, HER2/*neu* stimulated the transcriptional activity of the putative IKKβ promoter through ILK and Akt. Our results demonstrate that upregulation of IKKα and IKKβ by the ILK/Akt pathway is required for the HER2/*neu*-mediated NF-κB antiapoptotic pathway.**

*Oncogene* advance online publication, 15 March 2004; doi:10.1038/sj.onc.1207485

**Keywords:** HER2/*neu*; ILK; PI-3K; Akt; TNF-α; antiapoptotic pathway; IKK

Upon environmental stress, the fate of a cell depends on the balance between apoptotic and survival signals. Activation of receptor tyrosine kinases (RTKs) and the integrin family of adhesion receptors contributes to cell survival (Morino *et al.*, 1995; Zhu and Assoian, 1995; Downward, 1998; Datta *et al.*, 1999). A synergism between these two types of receptors in the survival signaling has also been shown (Sundberg and Rubin, 1996; Renshaw *et al.*, 1997; Gambaletta *et al.*, 2000). The β1 integrin cytoplasmic domain-binding protein, integrin-linked kinase (ILK), is crucial for the integrin-mediated cell survival pathway. ILK overexpression blocks anoikis (Hannigan *et al.*, 1996; Radeva *et al.*, 1997). It inhibits apoptosis by activating Akt, an essential downstream target of many RTKs (Cruet-Hennequart *et al.*, 2003; Gary *et al.*, 2003). Thus, ILK may play an important role in the crosstalk between RTK and integrin in the cell survival pathway.

The transcription factor nuclear factor kappa-B (NF-κB) is a major inducer of stress-responding genes in the survival pathway. NF-κB is activated by diverse stimuli, such as tumor necrosis factor-α (TNF-α), chemotherapeutic agents, γ-radiation, and growth factors, etc. RTKs like EGFR and HER2/*neu* (Karin *et al.*, 2002) can also promote cell survival through NF-κB activation. Upon stimulation, NF-κB upstream molecules, IκB kinase (IKK)α, β, and γ, are phosphorylated by NIK (Malinin *et al.*, 1997; Ling *et al.*, 1998), MEKK1 (Lee *et al.*, 1998; Nakano *et al.*, 1998), or Akt (Ozes *et al.*, 1999; Romashkova and Makarov, 1999; Zhou *et al.*, 2000), and form a triad complex. This IKK complex then phosphorylates IκB, the NF-κB-sequestering molecule, resulting in its degradation. Subsequently, NF-κB is released and translocates to the nucleus to activate transcription (Palombella *et al.*, 1994; Thanos and Maniatis, 1995).

HER2/*neu* is overexpressed in many types of tumors and is related to advanced malignancy. A point mutation in the transmembrane region of HER2/*neu* causes its constitutive activation. This constitutively active HER2/*neu* activates multiple downstream pathways (e.g. AP-1, NF-κB, E2F-1, etc.), causing malignant transformation (Galang *et al.*, 1996; Hung and Lau, 1999; Lee *et al.*, 2000), and inducing cellular resistance to therapeutic agents (Yu and Hung, 2000; Tan *et al.*, 2002b). We have previously reported that HER2/*neu* blocked TNF-α-induced apoptosis through the activation of the PI-3K/Akt/NF-κB pathway (Zhou *et al.*, 2000). In this study, we investigated the role that ILK plays in the HER2/*neu*-induced cell survival pathway.

To determine the effect HER2/*neu* has on ILK, we examined ILK kinase activity and protein expression in NIH3T3, and the HER2/*neu*-transformed NIH3T3 (3T3-HER2) cells using an immunocomplex kinase

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[4]These two authors contribute equally in this work
[5]Predoctoral fellow of DOD breast cancer Grant no. DAMD17-02-1-04541
[6]Predoctoral fellow of DOD breast cancer training Grant no. DAMD17-99-9264
Received 24 October 2003; revised 26 December 2003; accepted 5 January 2004

assay and Western blot, respectively. ILK kinase activity, as shown by the myelin basic protein phosphorylation (p-MBP) levels, was significantly upregulated in 3T3-HER2 cells (Figure 1a, lane 3), while there was no significant difference in the ILK protein levels in the two cell lines (Figure 1b). This indicated that HER2/*neu* expression enhances ILK kinase activity without increasing its expression. Since it has been shown that PI-3K activates ILK (Delcommenne *et al.*, 1998), and PI-3K is downstream of HER2/*neu*, we therefore asked whether HER2/*neu*-induced ILK activity required PI-3K. 3T3-HER2 cells were treated with the RTK inhibitor emodin and PI-3K-specific inhibitor wortmannin, and then ILK activity was examined. Both inhibitors suppressed ILK kinase activity (Figure 1c, lanes 2 and 3) in 3T3-HER2 cells. However, only emodin inhibited tyrosine phosphorylation (PY20) of HER2/*neu*. This indicates that the RTK activity of HER2/*neu* is required to activate PI-3K and ILK, and confirms that ILK is downstream of PI-3K. As a control, we also determined the effects of the inhibitors on Akt, the molecule immediately downstream of PI-3K in the cell survival pathway. As expected, Akt phosphorylation (p-Akt) was also blocked by emodin (Figure 1c, lane 3) and wortmannin (Figure 1c, lane 2), while its protein level was unchanged (α-Akt). These data suggest that HER2/*neu* induces ILK activity through PI-3K.

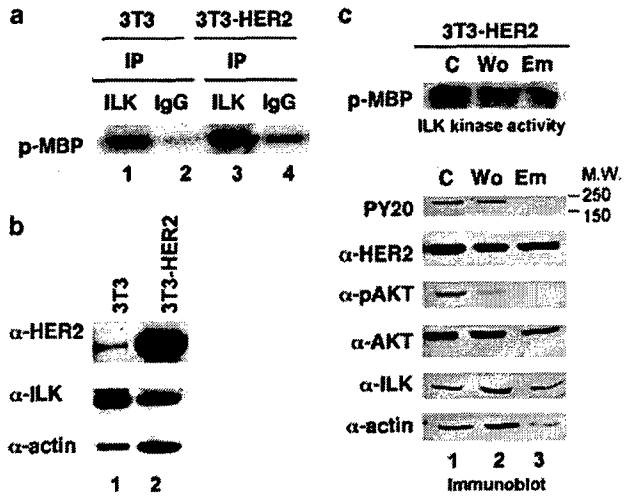


**Figure 1** HER2/*neu* activates the kinase activity of ILK. (**a**) To determine ILK kinase activity in NIH3T3 and 3T3-HER2 cell lines, cells were lysed with immunoprecipitation (IP) buffer (0.5% NP-40, 150 mM NaCl, 50 mM Tris (pH 8.0), 2% aprotinin, 5 mM PMSF, 100 mM NaF, 2 mM $Na_3VO_4$, and 1 mM EDTA). Protein (500 μg) from each sample was incubated at 4°C with 1 μg of antibody or control rabbit IgG overnight and for another 2 h after the addition of protein A–agarose. The immunoprecipitates were washed with IP buffer and kinase buffer (20 mM HEPES (pH 7.4), 150 mM KCl, 5 mM $MnCl_2$, 5 mM NaF, 1 mM DTT), and then resuspended in 40 μl of kinase buffer containing 5 μg of MBP as kinase substrate and 10 μCi of [γ-$^{32}$P]ATP. Following 30 min incubations at 30°C, the reactions were terminated with 40 μl of 2 × Laemmli SDS sample buffer. Samples were incubated for 5 min at 96°C and resolved by 15% SDS–PAGE. The kinase activity of the IPs was indicated by the amount of $^{32}$P labeling of MBP (p-MBP). (**b**) To determine the level of ILK protein expression in NIH3T3 (3T3) and 3T3-HER2 cell lines, protein lysates from the two cell lines were analysed by Western blotting with anti-HER2 (α-HER2), anti-ILK (α-ILK), and anti-β-actin (α-actin) antibodies. Equal amounts of protein from cell lysates were separated on 8% polyacrylamide gels and transferred to nitrocellulose filters. Each filter was blocked at room temperature for 1 h with 5% milk in PBS containing 0.05% Tween-20. They were then incubated at 4°C overnight in the same solution with anti-ILK antibody (1 : 500) (Boehringer-Mannheim Corp., Indianapolis, IN, USA) and anti-Her2/*neu* antibody (1 : 2000). The filters were washed and incubated with horseradish peroxidase-conjugated secondary antibody for 40 min. Specific proteins were detected using an enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL, USA). (**c**) Tyrosine kinase inhibitor (emodin, Em) and PI-3K inhibitor (wortmannin, Wo) inhibit ILK activity. 3T3-HER-2 cells were treated with or without emodin (40 μM), or with or without wortmannin (100 nM) for 18 h. The kinase activity of immunoprecipitated ILK (upper panel) was indicated by the amount of $^{32}$P labeling of MBP (p-MBP), as in (**a**). The same samples were analysed by Western blotting (lower panel) as described in (**b**). The molecular weight (MW) marker for PY20 blotting was labeled to indicate its position corresponding to HER2/*neu*

HER2/*neu* induces resistance to various apoptotic stimuli such as TNF-α treatment. To explore the role of ILK in HER2/*neu*-induced resistance to TNF-α treatment, we established two stable kinase-deficient mutant ILK (ILK-KD) transfectant clones of 3T3-HER2 cells, namely, 3T3-HER2-KD-1 and KD-2 (referred as KD-1 and KD-2). As expected, 3T3-HER2 cells were more resistant to TNF-α-induced apoptosis than parental NIH3T3 cells. However, the two ILK-KD clones were much more sensitive to TNF-α treatment than either NIH3T3 or 3T3-HER2 cells (Figure 2a). The role of ILK in the HER2/*neu*-induced survival pathway was further examined in an anoikis assay. NIH3T3, 3T3-HER2, and KD-1 and KD-2 cells were cultured in suspension conditions, and then their apoptotic profiles were analysed. Similar to TNF-α-induced apoptosis (Figure 2a), 3T3-HER2 cells were resistant to anoikis-induced apoptosis as compared with the parental NIH3T3 cells, and ILK-KD clones were much more sensitive under the same conditions (Figure 2b). These results indicate that ILK is required for HER2/*neu*-induced survival signaling, which leads to resistance to TNF-α and anoikis. Previously, we have shown that HER2/*neu* antagonizes TNF-α-induced apoptosis by activating NF-κB. To determine whether ILK is involved in this mechanism, the transcriptional activity of NF-κB in these cell lines was examined by a luciferase reporter assay. TNF-α-induced transcriptional activity of NF-κB in 3T3-HER-2 cells was stronger than that in NIH3T3 cells, as expected. However, this induction was almost completely suppressed in the KD-1 and KD-2 cells (Figure 2c). Taken together, these results indicate that ILK activity is required for HER2/*neu*-induced NF-κB activation, and blockage of ILK activity sensitizes cells to TNF-α and anoikis.

To determine how ILK regulates NF-κB activity, we examine the effects of ILK-KD on the molecules upstream of NF-κB. IκB binds to NF-κB and sequesters it in the cytoplasm. Upon TNF-α stimulation, the IKK complex phosphorylates I-κB, leading to its degradation and NF-κB activation. TNF-α treatment enhanced the degradation of IκB-α in NIH3T3 and 3T3-HER2 cells,

but not in KD cells (Figure 3a), suggesting that ILK-KD inhibits NF-κB activity through the stabilization of IκB-α. We then examined the effect ILK-KD had on the IKK complex in the three cell lines, since it is directly upstream of IκB. Unexpectedly, we found that IKKα (α-IKKα) and IKKβ (α-IKKβ) protein levels were dramatically upregulated in 3T3-HER2 cells (Figure 3b, lane 3) relative to parental NIH3T3 (Figure 3b, lane 2) cells, and ILK-KD suppressed HER2/*neu*-induced IKKα and IKKβ protein levels in KD (Figure 3b, lane 3) cells. These results indicate that HER2/*neu* induces the expression of IKKα and IKKβ through ILK.

To explore the mechanism of ILK regulation of IKKα and IKKβ expression, we performed a Northern blot analysis. The mRNA levels of both IKKα and IKKβ were upregulated in 3T3-HER2 cells (Figure 3c, lane 2) relative to NIH3T3 cells (Figure 3c, lane 3), but were suppressed in 3T3-HER2-KD cells (Figure 3c, lane 1). This result, which is consistent to the pattern of IKKα and IKKβ protein level in Figure 3b, suggests that HER2/*neu* upregulates the mRNA expression of IKKα and IKKβ through ILK. It has been shown that ILK can activate Akt, a crucial mediator of survival in mammalian cells (Tan *et al*., 2002a). Therefore, we next explored whether Akt was involved in the ILK-induced expression of IKKα and IKKβ using a stable dominant-negative Akt (dnAkt) clone of 3T3-HER2 (3T3-HER2-dnAkt) described previously (Zhou *et al*., 2001). HER2/*neu*-induced IKKα and IKKβ expression were suppressed in 3T3-HER2-dnAkt cells as well as in KD cells (Figure 4a, lane 3 and 4, respectively) compared to parental 3T3-HER2 (Figure 4a, lane 2). It should be mentioned that dnAkt inhibits Akt activity through competition for downstream substrates, and therefore does not affect the phosphorylation status of Akt (Figure 4a, lane 2 and 3) (Zhou *et al*., 2001). However, Akt phosphorylation (α-pAkt in Figure 4a) was reduced in KD cells (Figure 4a, lane 4) compared with parental 3T3-HER2 cells (Figure 4a, lane 2), while the protein expression level of Akt is not influenced by HER2/*neu* status in these cell lines (Figure 4a, α-Akt). The higher level of Akt blotting in 3T3-HER2-dnAkt cells came from the overexpression of dnAkt. These results indicate that both ILK and Akt activation are required for HER2/neu-induced IKKα and IKKβ expression. Together with the fact that ILK can phosphorylate Akt, the results also suggest that HER2/*neu*-mediated IKKα and IKKβ upregulation involves ILK-induced Akt activity.

Since the mRNA levels of IKKα and IKKβ were enhanced by HER2/*neu* (Figure 3c), we tested whether the HER2/ILK/Akt pathway may activate transcription of IKKβ using the putative IKKβ promoter, a 567-bp

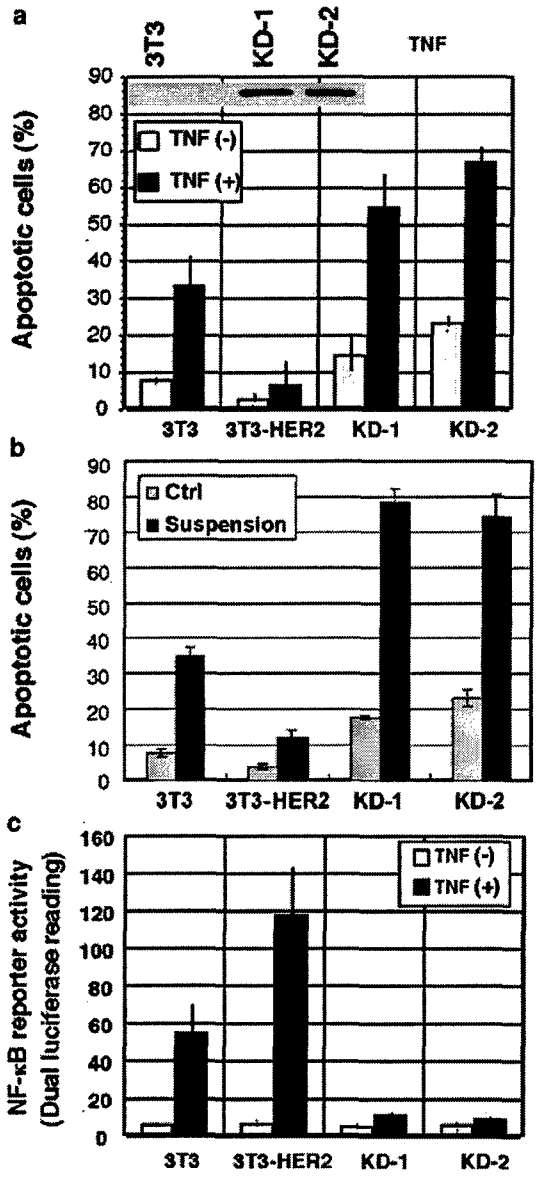


**Figure 2** ILK-KD inhibits HER2/*neu*-induced antiapoptotic and NF-κB activity. (**a**) ILK-KD sensitizes cells to TNF-α-induced apoptosis. Expressions of ILK-KD proteins in 3T3-HER2-KD clones (KD-1 and KD-2) were determined by Western blotting using anti-V5 tag antibody (Invitrogen, Inc., CA, USA), as shown in the inset figure. Cells were treated with TNF-α (TNF+) or left untreated (TNF−) for 48 h, and then the apoptotic cell ratio were calculated as sub-G1 population determined by flow cytometry. Data are presented as the means of three independent experiments with standard deviation. (**b**) ILK-KD sensitizes cells to anoikis. Cells were grown in DMEM/F12 medium containing 10% fetal bovine serum for 24 h and then plated on attached surface (ctrl) or poly-HEMA-coated plates (10 mg/ml) with 1% serum for 48 h. Cells were collected and apoptosis was analysed by a flow cytometry assay. The apoptotic cell ratios were calculated as sub-G1 population determined by flow cytometry. (**c**) ILK-KD inhibits the activity of NF-κB induced by TNF-α. NIH3T3, 3T3-HER2, and 3T3-HER2-KD (KD-1 and KD-2) cells were cotransfected with the pTK luciferase vector and either wild-type (WT) or mutant (MUT) NF-κB-responsive luciferase vector using the cationic liposome method in six-well plates. After 48 h of transfection, cells were incubated with (+) or without (−) TNF-α (20 ng/ml) for another 12 h. Cells were lysed with 1 × lysis buffer (Promega, Madison, WI, USA) and the luciferase activity was measured and normalized with the Dual luciferase assay kit (Promega). The WT/MUT ratio was calculated to represent the transcriptional activity of NF-κB

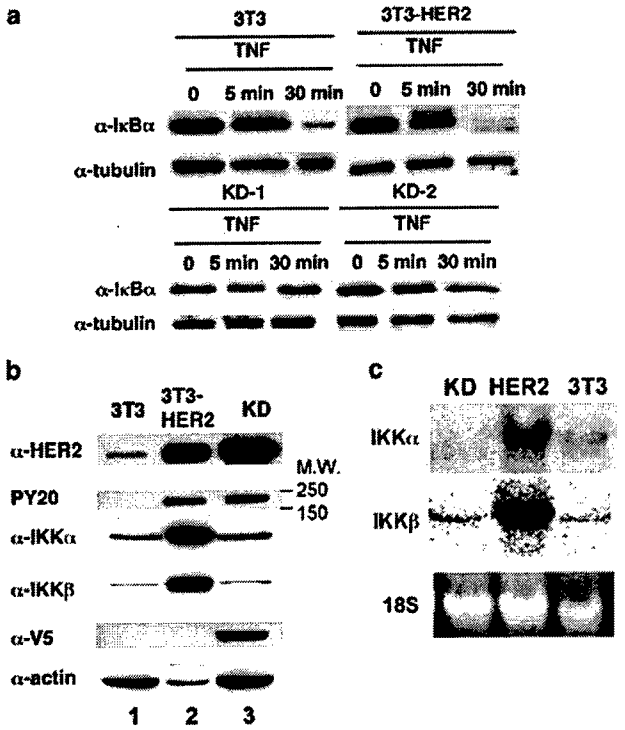


**Figure 3** ILK is required for IκB-α degradation and IKKα/IKKβ upregulation. (**a**) ILK-KD inhibits TNF-α-induced degradation of IκB-α. NIH3T3 (3T3), 3T3-HER2 cells, and 3T3-HER2-KD (KD-1 and KD-2) were cultured for 18 h without serum and treated without (0) or with TNF-α (40 ng/ml) for 5 or 30 min. Protein levels of IκB-α were analysed by Western blotting using anti-IκB-α antibody (α-IκBα) (Santa Cruz Biotech., Inc., CA, USA) as described in Figure 1a. β-tubulin (α-tubulin), blotted by anti-β-tubulin antibody (Santa Cruz Biotech., Inc., CA, USA), was used as a protein loading control. (**b**) ILK-KD blocked the HER2/*neu*-induced IKKα and IKKβ. Cell lysates prepared from NIH3T3 (3T3), 3T3-HER2, and one 3T3-HER2-KD clone (KD) were analysed by Western blotting. HER2/*neu* (α-HER2), ILK-KD (α-V5), and β-actin (α-actin) were blotted by the corresponding antibodies as described in Figures 1a and 2a. IKKα (α-IKKα) and IKKβ (α-IKKβ) were blotted by anti-IKKα and anti-IKKβ antibody (Cell Signaling Technology, Beverly, MA, USA), respectively. Tyrosine-phosphorylated (p-Tyr) HER2/*neu* was detected as p-Tyr protein of 185 kDa (the size of HER2/*neu*) by using anti-p-Tyr antibody PY20 (Santa Cruz Biotech., Inc., CA, USA). The molecular weight (MW) marker for PY20 blotting was labeled to indicate its position corresponding to HER2/*neu*. (**c**) Northern blotting analysis of IKKα and IKKβ in NIH3T3 (3T3), 3T3-HER2, and 3T3-HER2-KD cells. Total RNA was isolated from cultured cells using the Trizol (Gibco-BRL) method according to the manufacturer's instruction. RNA (10 μg) was separated by electrophoresis on a 1% agarose gel containing 0.6 M formadehyde and then transferred to blot membranes. The RNAs were UV crosslinked on the membranes, incubated in prehybridization buffer at 42°C, then hybridized overnight with $^{32}$P-labeled probes that were synthesized from cDNA expression vectors for IKKα and IKKβ (provided by Dr. Jong Deng in the authors' lab) using a random primed labeling kit (Boehringer Mannheim) according to the manufacturer's protocol. The hybridized membranes were washed in 2 × SSPE, then made visible and quantitated using a Molecular Dynamics Phosphoimager running ImageQuant software (Sunnyvale, CA, USA). The 18S ribosomal RNA (18S), stained with ethidium bromide, indicates loading control

5′-upstream flanking region (5′UFR) of the mouse IKKβ gene subcloned into a luciferase reporter vector. The promoter activity of the construct was tested in

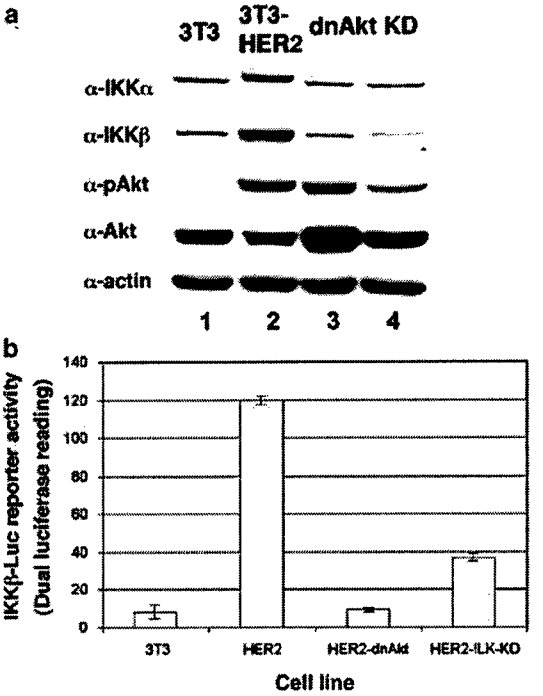


**Figure 4** HER2/*neu* upregulates IKKα and IKKβ through ILK and Akt. (**a**) Akt phosphorylation, which is required for HER2/*neu*-induced IKKα and IKKβ, was suppressed by ILK-KD. Lysates prepared from NIH3T3 (3T3), 3T3-HER2, 3T3-HER2-dnAkt (dnAkt), and 3T3-HER2-KD (KD) cells were analysed by Western blotting. IKKα (α-IKKα), IKKβ (α-IKKβ), and β-actin were blotted by the corresponding antibody as described in Figure 3b. Akt and Phospho-Ser 473-Akt (α-pAkt) were blotted by anti-Akt (Cell Signaling Technology, Beverly, MA, USA) and anti-phospho Akt antibody (New England Biolabs, Beverly, MA, USA), respectively. (**b**) HER2/*neu*-induced IKKβ promoter activity requires ILK and Akt. The 5′-UTR of IKKβ was PCR amplified using the mouse GenomeWalker Kit (Clontech, Palo Alto, CA, USA) with the designed primer UTR-1 (5′-AATTCCCGGGTAC-CAACACAATGGC-3′) and UTR-2 (5′-CCGCGAATTCCCGG-GTACCAACACA-3′) for primary and secondary PCR, respectively, according to the manufacturer's instruction. The secondary PCR product was cloned into pGL3-basic vector to get the reporter vector IKKβ-Luc. IKKβ-Luc was cotransfected with pTK luciferase vector into NIH3T3 (3T3), 3T3-HER2 (HER2), 3T3-HER2-dnAkt (HER2-dnAkt), and 3T3-HER2-KD (HER2-ILK-KD) cells. The transcriptional activity of IKKβ-Luc was determined by the Dual luciferase assay as described in Figure 2c

NIH3T3, 3T3-HER2, 3T3-HER2-dnAkt, and KD cells by a transient transfection and reporter assay. The putative IKKβ promoter was dramatically activated by HER2/*neu* (Figure 4b, HER2), and the activation was suppressed by dnAkt (Figure 4b, HER2-dnAkt) and ILK-KD (Figure 4b, HER2-ILK-KD), indicating that ILK and Akt were required for HER2/*neu*-mediated transcriptional activation of IKKβ. Taken together, these data suggest a novel mechanism by which HER2/*neu* upregulates the mRNA expression of IKKα and IKKβ through ILK-induced Akt activation, resulting in NF-κB activation.

In this study, we showed that ILK mediated HER2/*neu*-induced survival signaling, providing a link between

HER2/*neu* and integrin pathways. Previously, we have shown that HER2/*neu* constitutively activates NF-κB activity through the PI-3K/Akt pathway, causing resistance to TNF-α (Zhou *et al.*, 2000). Here, we demonstrated that ILK is required for the activation of NF-κB by HER2/*neu*, and identified a novel mechanism of HER2/*neu*-mediated IKK activation, namely, upregulation of IKKα and IKKβ through ILK and Akt. In conclusion, our study identifies a novel signal cascade, HER2/neu → PI-3K → ILK → Akt → IKKα/IKKβ transcription, which links the HER2/*neu*-mediated resistance to anoikis- and TNF-α-induced apoptosis. Therefore, together with our previous publication (Zhou *et al.*, 2000), there are two distinct mechanisms in which Akt may regulate the HER2/*neu*-mediated antiapoptotic pathway.

**Acknowledgements**
We thank Dr Shoukat Dedhar (University of British Columbia, British Columbia, Canada) for providing the ILK and ILK-KD plasmids. This work is supported by NIH PO1 CA 99031 and RO1 58880 (to M.-C.H.).

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APPENDIX 2-- DAMD17-99-1-9264

**Three Figures and Figure Legends**
**Kan-Hsueh Lan, M.D. – predoctoral trainee**
**Peptide/Protein Delivery System Targeting erbB2-overexpressing Breast Cancers**

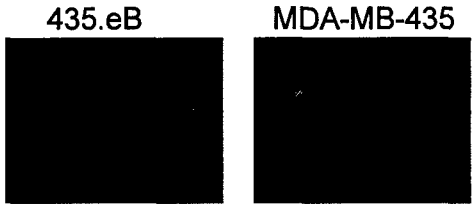


**Figure 1. TAT-AHNP-αStat3 peptide preferentially translocated to erbB2-overexpressing breast cancer cells.** Breast cancer cell lines MDA-MB-435 and the ErbB2 transfectant 435.eB were seeded on Chamber Slides. After 15 mins of 20 μM fluoresceine-TAT-AHNP-αStat3 treatment, cells were fixed and observed under fluorescent microscope. The subcellular localization of TAT-AHNP-αStat3 peptides was shown in green.

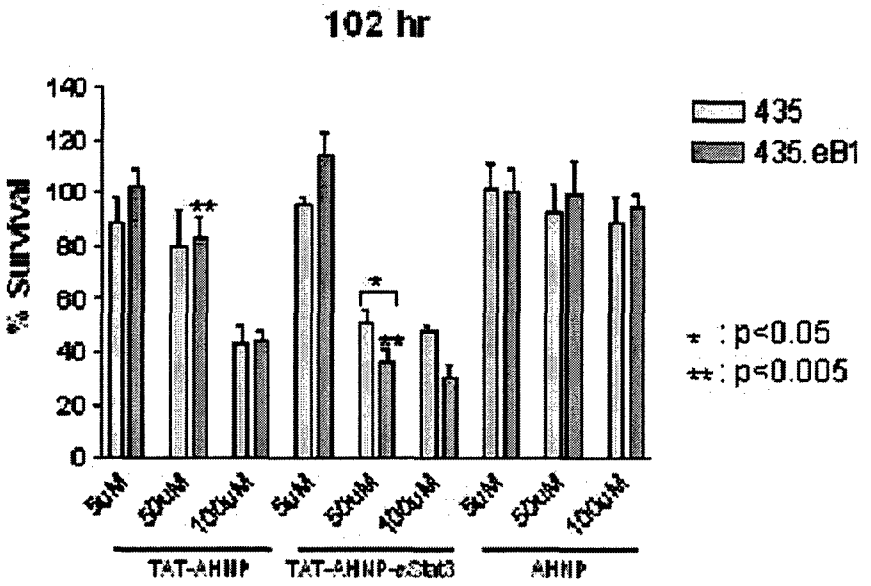


**Figure 2. TAT-AHNP-αStat3 peptide inhibited breast cancer cell growth *in vitro*.** Different concentrations (5, 50, or 100 μM) of peptides were applied to MDA-MB-435 or 435.eB cells in culture. The viability of cells was measured after 54 (not shown) and 102 hours of peptide treatment, respectively, using MTS assay.

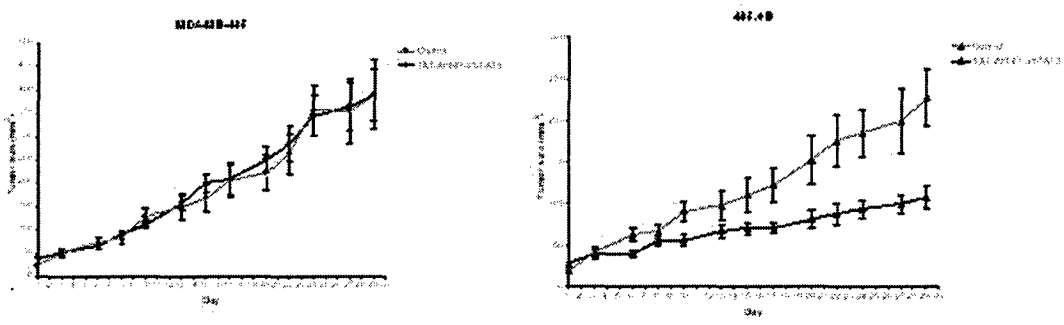

**Figure 3. TAT-AHNP-αStat3 peptide preferentially inhibited erbB2-overexpressing breast cancer cells *in vivo*.** Tumor xenografts of MDA-MB-435 and 435.eB breast cancer cells were established in the mammary fat pads (MFPs) on either side of 24 female SCID mice. The mice were randomly divided into two groups (12 mice in each group), and when the tumors became palpable (~30mm$^3$ of volume), we began to intra-peritoneally (i.p.) inject each group of mice with 15 nmole/mouse of TAT-AHNP (control) or TAT-AHNP-αStat3 peptides, respectively, three times per week. Tumor diameters were serially measured with calipers and tumor volumes were calculated using the follow formula: volume = width$^2$ × length/2.

# APPENDIX 3-- DAMD17-99-1-9264

## Four Figures and Figure Legends

Dung-Fang Lee, M.S. – predoctoral trainee

## A potent proapoptotic hybrid gene, tBid and Bax/Bak, in breast cancer

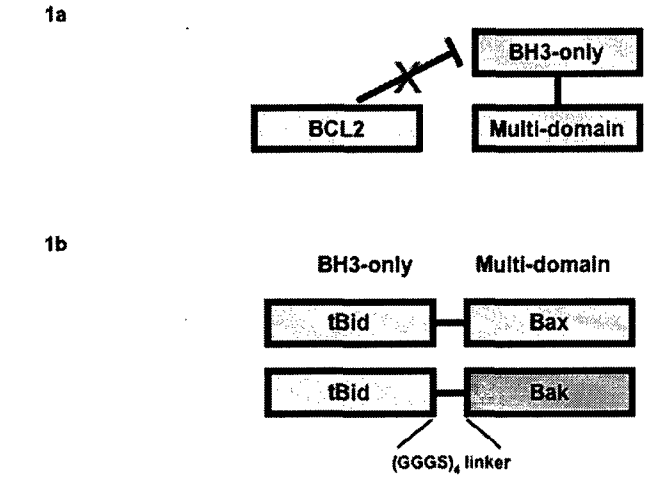


**Fig1. (a) The proposed model of hybrid gene prevents binding to Bcl2. (b) The schematic of two hybrid genes**

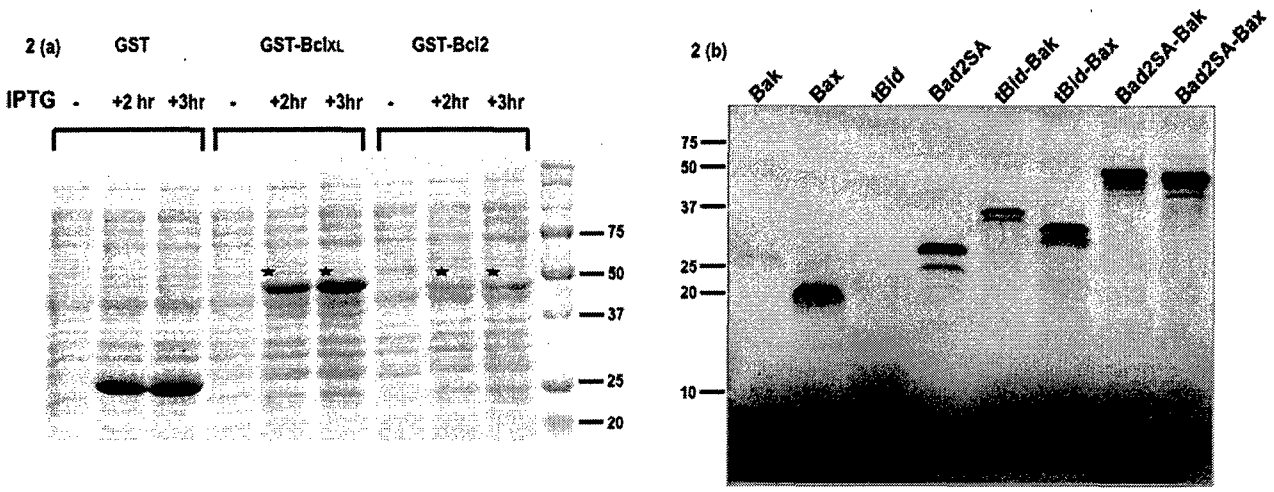


**Fig 2 (a) IPTG induces GST-Bcl2 and GST-BclXL expression.** To express these fusion proteins, colonies containing each recombinant plasmid were grown up overnight at 37°C in 2X YTA broth with 100 mg/ml ampicillin (Sigma). Fusion protein expression was induced by the addition of 0.2mM IPTG (Sigma) into the cultures for 2 hrs and 3 hrs at 30°C, respectively. Cells were collected and lysed by sampling buffer. Protein separations were carried out by SDS–PAGE and visualized by Coomassie blue staining. ∗ indicates the IPTG-induced proteins. **(b) The proapoptotic proteins (Bak, Bax, tBid, Bad2SA, tBid-Bak, tBid-Bax, Bad2SA-Bak, and Bad2SA-Bax) are produced by $^{35}$S labeled *In vitro* transcription and translation.** For in vitro transcription and translation, 1 µg of the full-length MPP-1 cDNA was used for coupled in vitro transcription and translation with a rabbit reticulocyte lysate kit (Promega, Madison, WI) in the presence of T7 RNA polymerase, rabbit reticulocyte lysate, and [$^{35}$S]methionine (trans $^{35}$S label; ICN, Irvine, CA). Translation was carried out at 30 °C for 1 hour followed by SDS-PAGE of a 5 µl aliquot to confirm the presence of translation products.

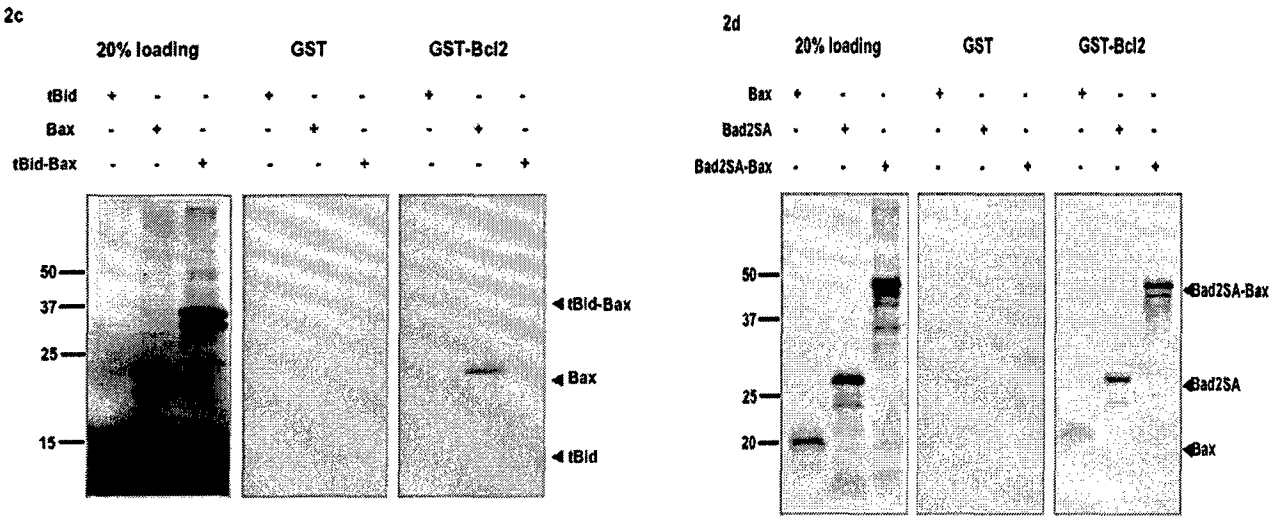


**Fig2 (c) the hybrid proapoptotic gene tBid-Bax exhibits weaker binding affinity with GST-Bcl2 *in vitro*.** GST and GST-Bcl2 are purified by glutathione-sepharose beads (Pharmacia) and used to pull down the $^{35}S$ labeled tBid, Bax and tBid-Bax protein. **(d) The hybrid proapoptotic gene Bad2SA-Bax retains the binding affinity with GST-Bcl2 *in vitro*.** GST and GST-Bcl2 are purified by glutathione-sepharose beads (Pharmacia) and used to pull down the $^{35}S$ labeled Bad2SA, Bax and Bad2SA-Bax protein.

3

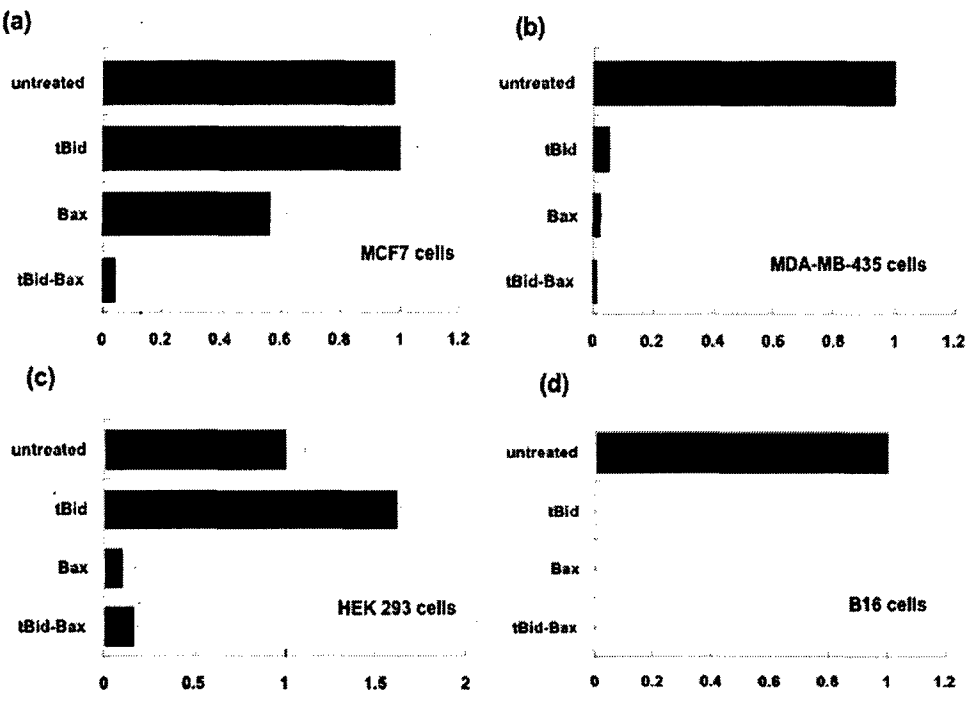


**Fig 3. The expression of the tBid-Bax hybrid gene exhibits a stronger growth-inhibitory activity than tBid/Bax does in various cancer cell lines.** *A* comparison of the growth-inhibitory activity between the tBid, Bax and tBid-Bax in different human cancer cell lines. Human breast cancer cell lines MCF-7 and MDA-MB-435, the human kidney cell line HEK293, and the mouse lung cancer cell line B16 were cotransfected with 50 ng of CMV-*Luc* and 2 μg of tBid, Bax, and tBid-Bax. The relative activities were calculated by setting the *Luc* activities obtained from transfections with vector (untreated) at 100%. The data represent means of three independent experiments; *bars*, SD., significant difference compared with wt ($P < 0.05$).

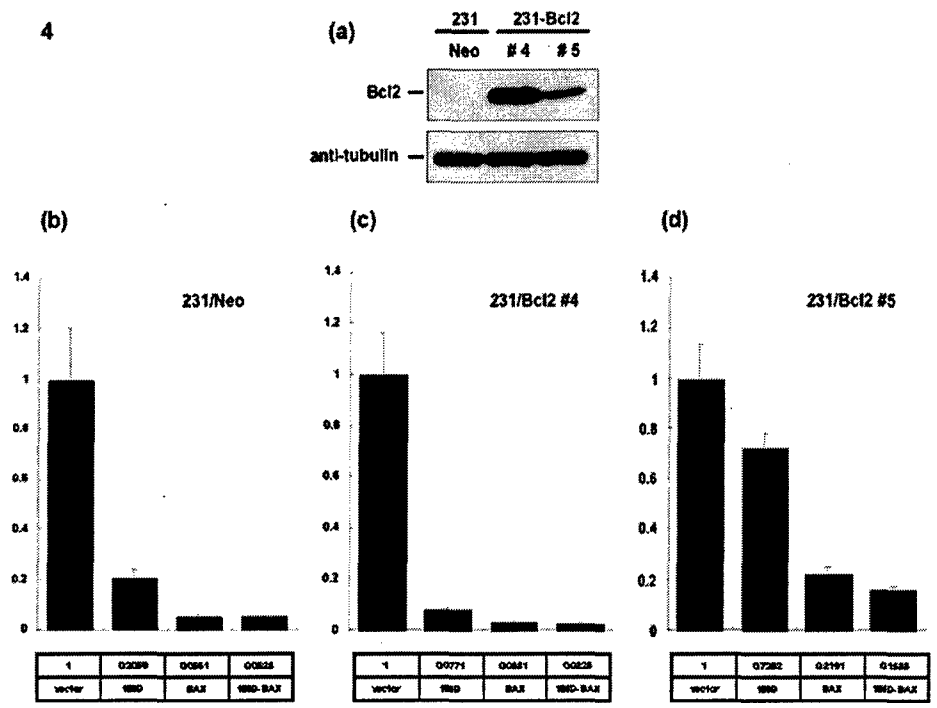


**Fig 4. The expression of the tBid-Bax hybrid gene exhibits a stronger growth-inhibitory activity than tBid/Bax does in Bcl2-overexpressed MDA-MD-231 breast cancer cells.** Human breast cancer cell line MDA-MB-231#neo, MDA-MB-231#4, and MDA-MB-231#5, were cotransfected with 50 ng of CMV-*Luc* and 2 μg of tBid, Bax, or tBid-Bax. The relative activities were calculated by setting the *Luc* activities obtained from transfections with vector at 100%. The data represent means of three independent experiments; *bars*, SD., significant difference

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